치의과학박사 학위논문

Short- and long-term effects of botulinum toxin A injection into the masseter muscle of rats: immunohistochemical and ultrastructural study

백서의 교근에 보툴리눔 독소 A 주사 시
단기 및 장기간의 영향: 면역조직학적 및 초미세구조 연구

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서울대학교 대학원
치의과학과 치과교정학 전공
문 영 민

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-ABSTRACT-

Short- and long-term effects of botulinum toxin A injection into the masseter muscle of rats: immunohistochemical and ultrastructural study

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Introduction: In the head and neck area of human, botulinum toxin A (BTX) injections into the masticatory muscle are used for several indications such as trismus, bruxism, clenching, migraine, temporomandibular joint disorders or masseter hypertrophy. For more evidence-based applications of BTX injection into the masseter muscle, well-designed randomized clinical trials and basic studies about the responses and the recovery of muscle tissues for a long time after BTX injection are more needed. The purpose of this animal model study was to investigate the atrophy and recovery of masseter muscle following bilateral BTX injection using two different dose of BTX in the short and long term.

Materials and Methods: Mature male rats (n=30) were randomized into 3 groups. Rats of each group received saline (control group), 5 units of BTX (5 U BTX group) or 10 units of BTX (10 U BTX group) into the each masseter muscle bilaterally. The amount of daily food intake and body weight was measured until 12 weeks after the injection. The thickness of the masseter muscle was also measured using ultrasonography weekly. A half of animals were sacrificed at 2 weeks after the injection. The other animals were sacrificed at 12 weeks after injection. Specimens of the injected masseter muscle were processed for immunohistochemical determination of myosin heavy chain (MyHC) composition and ultrastructural analysis.

Results: There was no statistically significant difference among groups in the body weight and the amount of food consumption after 2 weeks. The maximal atrophy of masseter muscle after BTX injection was found at 6 weeks. A longer time than 12 weeks was required for BTX injected masseter muscle to recover completely. There was no significant difference in muscle thickness according to the injected dose of BTX. The expression level of MyHC type I and type IIa in the BTX treated masseter muscles was significantly increased at 2 weeks. However, the expression level of MyHC type I and type IIa was not different from that of the control group after 12 weeks regardless of the injected dose of BTX. Abnormalities of myofilaments were observed in both BTX treatment groups at 12 weeks. The mitochondrial swelling and alteration of cristae were prominent in the 10 U BTX group after 12 weeks.

Conclusions: BTX injection led to changes in the MyHC composition in the short term, but the MyHC composition was almost recovered after 12 weeks. BTX injection to the masseter muscle of rats with a high dosage might be associated with increased mitochondrial susceptibility to apoptosis as a delayed phenomenon.

Key words: botulinum toxin A, masseter muscle, myosin heavy chain, ultrastructural study
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Ⅰ. INTRODUCTION

Botulinum toxin A (BTX) is a protein derived from Gram (-) anaerobic bacterium *Clostridium botulinum*. It is a neurotoxin that inhibits release of neurotransmitter in cholinergic nerve endings. Acetylcholine vesicles are blocked at the presynaptic membrane because BTX enzymatically cleaves the synaptosomal-associated protein of 25kDa (SNAP-25) required for vesicle fusion and release of acetylcholine at the axon end.[1-3] With the inhibition of neurotransmitter release into the synaptic cleft, BTX paralyses the neuromuscular junctions and autonomic synapses of glands.

In the head and neck area of human, BTX injection is used in masticatory muscles for several indications such as trismus, bruxism, clenching, temporomandibular joint disorders or masseter hypertrophy.[4-10] It is also used in facial injections for the treatment of sialorrhoea, blepharospasm and hemifacial spasm and for the cosmetic purposes.[4, 11-13] Recently, the therapeutic uses of BTX have expanded exponentially to include a wide range of medical and surgical conditions. This has been aided by a greater understanding of its underlying physiology as well as improved efficacy and safety.[4]

In orthodontics, weak masticatory musculature, decreased occlusal force, and a tendency for extrusion have been associated with malocclusions characterized by increased anterior facial height and high mandibular plane angles.[14, 15] Whereas the use of BTX in these patients might exacerbate this extrusive tendency, it can be useful in patients with a decreased lower facial height and large musculature, in which extrusion would be beneficial but difficult because of their strong biting forces.[16] With the use of botulinum toxin instead of removable functional appliance such as anterior bite plane for orthodontic patients who have deep bite or clenching habit, the time required for the orthodontic treatment can be reduced, and patients would be far more comfortable and functional (eating, speaking and swallowing).[17]

For more evidence-based applications of BTX injection into the masseter muscle, well-designed randomized clinical trials and basic studies about the responses and the recovery of muscle tissues for a long time after BTX injection are more needed.[18, 19] There have been several reports to investigate the effect of BTX injection into the masseter muscle in the animal model.[16, 20-26] But these studies have limitations that are unilateral BTX injection into the masseter muscle unlike bilateral injections used in the most clinical cases, lack of consideration for BTX dosage difference, and only single observation period of either short- or long-term.

In our previous study, we evaluated the early effect after bilateral BTX injection into the masseter muscle of rats with functional and histological evaluation methods.[27] The purpose of this study was to investigate the atrophy and recovery of masseter muscle following BTX injection using two different dose of BTX in the short and long term.

Ⅱ. REVIEW OF LITERATURE

1. Overview on botulinum toxin

1) History[11, 28]

The idea for a possible therapeutic use for botulinum toxin was first developed by the German physician Justinus Kerner (1786-1862). He deduced that the toxin acted by interrupting signal transmission within the peripheral sympathetic nervous system, leaving sensory transmission intact. He called the toxin a “sausage poison,” because it was observed that illness followed ingestion of spoiled sausage. In 1870, John Muller, another German physician, coined the name “botulism” (from the Latin root botulus, which means “sausage”). Émile van Ermengem, professor of bacteriology and a student of Robert Koch, correctly described Clostridium botulinum as the bacterial source of the toxin. Thirty-four attendees at a funeral were poisoned by eating partially salted ham, an extract of which was found to cause botulism-like paralysis in laboratory animals. Van Ermengem isolated and grew the bacterium, and described its toxin, which was later purified by P Tessmer Snipe and Hermann Sommer. In 1949, Burgen was the first to discover that the toxin was able to block neuromuscular transmission. Scott et al. proved this fact by experimentally administering the Type A strain in monkeys. This strain was approved by the US Food and Drug Administration (FDA) in 1989 under the trade name Botox (Allergan, Inc, Irvine, Calif) for treating strabismus, blepharospasm, and hemifacial spasm in patients younger than 12-year-old. In the year 2000, Botox was approved for use in treating cervical dystonia (wry neck) and 2 years later for the temporary improvement of moderate to severe frown lines between the eyebrows (glabellar lines). Serotype B has been FDA approved for treating cervical dystonia.

2) Mechanism of action

Botulinum toxin is composed of two peptide subunits, a heavy chain (100kDas) and a light chain (50kDas), which are held together by a disulfide bond. The action of botulinum toxin follows a 4-step mechanism including binding to the neuronal membrane, endocytosis, membrane translocation, and proteolysis of SNARE proteins.[1]

In its mechanism of action, the heavy chain of botulinum toxin is first used to find its neuronal targets and bind to the gangliosides and membrane proteins of presynaptic neurons. Next, the toxin is then endocytosed into the cell membrane. The heavy chain undergoes a conformational change important for translocating the light chain into the cytosol of the neuron. Finally, after the light chain of botulinum toxin is brought into the cytosol of the targeted neuron, it is released from the heavy chain so that it can reach its active cleavage sites on the SNARE proteins.[1] The light chain is released from the heavy chain by the reduction of the disulfide bond holding the two together. The reduction of this disulfide bond is mediated by the NADPH-thioredoxin reductase-thioredoxin system.[2] The light chain of botulinum toxin acts as a metalloprotease on SNARE proteins that is dependent on Zn(II) ions,[29] cleaving them and eliminating their function in exocytosis.

There are 7 known isotypes of botulinum toxin, botulinum toxin A to G, each with different specific cleavage sites on SNARE proteins. SNAP25, a member of the SNARE protein family located in the membrane of cells, is cleaved by botulinum toxin isotypes A, C, and E. The cleavage of SNAP-25 by these isotypes of botulinum toxin greatly inhibits their function in forming the SNARE complex for fusion of vesicles to the synaptic membrane. Botulinum toxin C also targets Syntaxin-1, another SNARE protein located in the synaptic membrane. It degenerates these Syntaxin proteins with a similar outcome as with SNAP-25. A third SNARE protein, Synaptobrevin (VAMP), is located on cell vesicles. VAMP is targeted and cleaved by botulinum toxin isotypes B, D, and F in synaptic neurons.[3]

In each of these cases, botulinum toxin causes functional damage to SNARE proteins, which has significant physiological and medical implications. By damaging SNARE

proteins, the toxin prevents synaptic vesicles from fusing to the synaptic membrane and releasing their neurotransmitters, in particular acetylcholine, into the synaptic cleft. With the inhibition of neurotransmitter release into the synaptic cleft, action potentials cannot be propagated to stimulate muscle cells. This result in paralysis of those infected and in serious cases, it can cause death. Although the effects of botulinum toxin can be fatal, it has also been used as a therapeutic agent in medical and cosmetic treatments.

3) Clinical uses of botulinum toxin in the dental conditions

Each vial of Botox contains 100 units (U) of C. botulinum type A neurotoxin complex, 0.5 mg of albumin (human), and 0.9 mg of sodium chloride in a sterile, vacuum-dried form without preservatives. Commercial preparations of botulinum toxin available in the United States include Botox (BTX type A, Allergan, Irvine, USA), Myobloc (BTX type B, Solstice Neurosciences, Inc., San Francisco, USA), and Dysport (BTX type A, Ipsen Ltd., Maidenhead, UK). In humans, the LD50 is estimated to be 40 U/kg (2800 U for a 70 kg adult). Botulinum toxin type A can be used in the following dental conditions.

(1) Temporomandibular disorders

Temporomandibular disorders (TMD) are a variety of disorders involving the temporomandibular joint, the soft tissue structures within the joint, and the mastication muscles. The most common type of painful TMD is myofacial pain, which is characterized by diffuse pain in the mastication muscles, along with muscle dysfunction and tightness. In the majority of patients with myofacial facial pain, the etiologic factors are muscular spasticity and fatigue secondary to microtrauma from malocclusion, bruxisms, hypermobility, external stressors, and individual psychomotor behaviors (e.g., excessive gum chewing).[30] Because of its paralyzing effect, BTX therapy is expected to decrease

stresses on the TMJ and related tissues and relieve the affected muscular component of TMD.[5, 31]

(2) Bruxism

Botulinum neurotoxin has shown promise in decreasing the symptoms of bruxism.[32] Ivanhoe et al. reported success with a 200 U dose of botulinum toxin Type A in a separate brain injury case report.[33] A long-term, open-label trial study with a history of severe bruxism who were refractory to medical and dental procedures, to them botulinum toxin Type A injections were given into the masseters (mean dose: 61.7 U/side; range 25 U to 100 U), which results in a total duration of therapeutic response of 19 weeks.[6]

(3) Clenching

Excessive forces created by parafunctional clenching impede healing and reattachment of gum and bone in the mouth after trauma.[34] Botulinum toxin type A limits the muscle contraction, and this reduction in clenching intensity will allow traumatized tissue to heal. Because parafunctional clenching contributes to periodontal trauma, botulinum toxin type A can limit clenching before and after periodontal surgery to improve healing.

Orthodontic treatments on patients who are clenchers or have a deep or crossed bite are prolonged if the vertical component of muscular force is greater than the force of the fixed or removable appliance. These cases often require the use of removable functional retainers in combination with regular fixed braces in an attempt to control the component of vertical force.[35] With the use of botulinum toxin, orthodontic treatment time can be reduced, and patients would be far more comfortable and functional (eating, speaking, swallowing).

(4) Migraine

BTX was approved by the FDA for the treatment of chronic migraines in 2010, and it is the only prophylactic therapy specifically for chronic migraines.[36] The mechanism by which BTX reduces migraine frequency and intensity is not completely understood. BTX is thought to inhibit overactive motor neurons and hyperexcitable sensory neurons and suppress peripheral and central sensitization of trigeminal sensory nerves around muscle trigger points. BTX is most effective when injected into an active trigger point or at sites of compression of a branch of the trigeminal nerve. These sites of anatomic compression have been identified through clinical experience as well as through anatomic dissection. Compression can be related to facial impingement, muscle contraction when there is an intramuscular component to the nerve, as well as blood vessel impingement of the nerve.

(5) Oromandibular dystonia

Oromandibular dystonia (OD) represents a focal dystonia whereby repetitive or sustained spasms of the masticatory, facial, or lingual muscles result in involuntary and possibly painful jaw movements.[37] BTX has been shown to be clinically superior to medical therapy, particularly in the treatment of focal dystonias.[38] Injection of BTX into the muscles of the floor of the mouth, the muscles of mastication, and the extrinsic muscles of the tongue yield great improvement in this subtype of dystonia by paralyzing the hyperactive dysfunctional group of muscles. However, a recent Cochrane review showed no significant difference in pain reduction at four weeks between those who received BTX for subacute and chronic neck pain and those who received placebo injections.[39]

(6) Masseteric hypertrophy

Masseteric hypertrophy is recognized as an asymptomatic enlargement of one or both masseter muscles. It is commonly associated with abnormal habits such as bruxism and

habitual clenching. There have been some reports showing that BTX injection into the masseter muscle can be used as an alternative noninvasive treatment for masseteric hypertrophy.[9, 81, 82] These studies have revealed atrophy of the hypertrophic muscles after BTX injection using clinical photographs, ultrasound, electromyography and computed tomography.[7, 9, 40]

(7) Sialorrhoea

Sialorrhoea may occur in neurological and other akinetic disorders such as Parkinson's disease and cerebral palsy. There are several RCTs where the efficacy of BTX injections to the parotid and/or submandibular glands in such patients has been demonstrated.[12, 41, 42] The effects last 3–6 months and can be repeated. Injections can also be used for sialorrhoea caused by salivary fistulas and sialadenitis.[43]

2. Botulinum toxin in masticatory muscles

1) Masticatory performance, biting force, muscle volume and EMG

BTX injection into masseter muscle induced only brief periods of problems with chewing and the return of normal function after a short time.[35, 44] This might have been due to compensatory hyperfunction of the other masticatory muscles such as temporalis muscle or medial pterygoid muscle to adjust to the masticatory fuction.[20, 45]

The clinical literature on BTX in the masseter muscle indicates that maximal atrophy of the masseter follows loss of electromyography by about 2 months and is still sometimes observable at 1 to 2 years.[40, 46] In one report, voluntary bite force was subjectively normal in 8 days even though symptom relief persisted for 8 weeks.[35] In the best documented study to date, bite force began to recover in 3 weeks when muscle volume was still decreasing; bite force was fully recovered at 3 months, when the muscle was at minimal volume.[44]

Although the compensating effect by other masticating muscle groups was reflected in the recovery of bite force after 3 weeks, the volumetry of other masticating muscles did not show any compensatory hypertrophy. It is not clear how an atrophied muscle can produce a normal bite force, but a possible explanation is that the subjects had simply learned to produce normal voluntary force by using untreated muscles.

2) Eruption rate

Eruption rate has not been studied systematically for BTX treatment in either human or animal models. When an opposing tooth is removed experimentally[47] or clinically,[48] eruption speeds up, and overeruption occurs. Muscle weakness in humans[49] and muscle resection in rats[50] have the same effect. Because BTX paralysis also causes muscle weakness, it can lead to supraeruption.

Interestingly, incisor eruption rate in rabbits was significantly decreased for the botulinum neurotoxin type A group, an effect attributed to decreased attrition. Although findings from ever-growing rabbit incisors cannot be extrapolated to human teeth, it is clear that botulinum neurotoxin type A caused a decrease in bite force that could influence dental eruption.[16]

3) Craniofacial growth and development

After BTX injection into the masseter muscle of growing rats, anthropometric measurements of bony structures attached to the masseter muscle such as ramus height, corpus length and mandibular plane angle showed a significant treatment effect.[23] In another study, The BTX injected group demonstrated smaller mandibular dimension compared with control group because of apoptosis at the proliferation stage of the reserve zone of the condylar cartilage in developing rat mandible.[26] In conclusion, after localized

masseter muscle atrophy induced by BTX injection, alterations of craniofacial bone growth and development could be seen.

4) Histomorphometric findings

The muscle fibers of saline injected masseter muscle of rats have a normal polygonal shape without necrosis or inflammation and the fiber size was uniform.[26] The muscle fibers of the BTX injected masseter muscle were more irregular and the nuclei were packed more densely than those of saline injected muscle. The formation of condensed myofibers without inflammatory reaction in the BTX injected muscle might be an expression of its degenerative atrophy.

In the study of adult rabits, the resulting underloading from BTX injection into the masseter muscle was sufficient to cause notable and persistent bone loss, particulary at the temporomandibular joint.[20] Another study also found that BTX in masticatory muscles of the adult rat induced bone loss at the condyle and alveolar regions of the mandible.[21]

In the ultrastructural study,[24] most fibres appeared normal in the masseter muscle of adult monkeys at 2 weeks after BTX injection. However, muscle fibres in the masseter muscle of monkeys showed myofibrillar dissolution, aberrations in the Z-line, and enlarged mitochondria in the region of the I-band at 4 weeks. In the 9-week and 12-week animals, the injected muscle was considerably smaller than the uninjected, contralateral muscle. Regions of the injected muscle contained fibers with markedly reduced cross-sectional area. Internalization of myonuclei, loss of myofibrillar organization, and helical complexes were common.

Ⅲ. MATERIALS AND METHODS

Animals and experimental design

Male Wistar rats aged 18 weeks were purchased from Samtako (Seoul, Korea). They were housed individually in controlled temperature (20–22°C) and hygrometry (around 40%) in a 12 h light: 12 h darkness cycle. They had free access to water. During the adaptation period (first week) all rats were fed ad libitum with a control semi-synthetic diet (4% lipids from soya vegetal oil, 74% carbohydrates from sucrose and cornstarch and 14% proteins from casein, supplemented with standard vitamins and mineral mix), following classical recommendations. All diets were prepared within Gangneung-Wonju National University facilities. All groups were maintained ad libitum for 7 days receiving a diet similar to the adaptation diet with measuring a daily spontaneous intake (26.1±4.1 g/d, n=30). At the end of the normal diet period, rats (20 weeks-old) were separated: the control group received a saline injection into both masseter muscles (group 1, n=10) and the others were separated in two groups for BTX injection study (n=10 per group). These two groups were assessed in order to compare the dose dependent effect of BTX injection on physiological parameters in 2 animal groups receiving re-feeding diets. All re-feeding diets were the same to the Ad libitum control period. In order to measure food intake all groups were individually housed. Group 1 was saline injected group. Group 2 was 5 units (U) BTX injection group to each masseter muscle. Group 3 was 10 units (U) BTX injection group to each masseter muscle. The point where BTX or saline was injected was halfway between anterior border and posterior border of masseter muscle, intersecting with midline between zygomatic arch and mandibular lower border. The amount of daily food intake and body weight was measured until 12 weeks after the injection. The thickness of the masseter muscle was also measured using ultrasonography (E-CUBE9, Alpinion medical systems, Seoul, South Korea). Measuring the masseter muscle thickness was performed before BTX injection as a baseline and until 12 weeks after BTX injection. The thickest part in the ultrasound image of masseter muscle was measured at lower 1/3 between zygomatic arch

and lower border of mandible in the ultrasonography. A half of animals were sacrificed at 14 days after the injection. The other animals were sacrificed at 12 weeks after injection. All procedures were conducted according to the guidelines of laboratory animal care and were approved by the Gangneung-Wonju National University for animal research.

Histomorphometric evaluation

The samples were harvested, decalcified in 5% nitric acid for 5 days, and dehydrated in ethyl alcohol and xylene. After separation of the calvarial bones, the head samples were embedded in paraffin blocks. The paraffin blocks of middle portion between zygomatic arch and mandibular lower border were horizontally sliced into sections that were then stained with hematoxylin and eosin. The section with the occlusal plane area was selected.

The staining procedure for hematoxylin and eosin staining was as follows. First, de-wax and hydrate paraffin sections. The slide was stained in hematoxylin for 5 min. Overstained sections can easily be differentiated by agitating for a second in acid-alcohol, then washing in tap water for 5 min. The slides were immersed in eosin for 30 seconds and then wash them in running tap water for 1 min. The slides were dehydrated and clear in xylene. A total of 30 slides were fabricated and examined.

Digital images of the selected sections were captured with a digital camera (DP-73; Olympus, Tokyo, Japan). The images were analyzed by Sigma Scan pro (SPSS, Chicago, IL).

Immunohistochemical determination of myosin heavy chain type I, myosin heavy chain type IIa and BCL2 in rat masseter muscle

To investigate the alteration of muscle fiber type after BTX treatment, immunohistochemical determination of myosin heavy chain type I (MyHC-I) and myosin

heavy chain type IIa (MyHC-IIa) was performed. The change of BCL2 associated with apoptosis after BTX treatment was also identified by using immunohistochemical staining. To determine the level of expression of myosin heavy chain type I, myosin heavy chain type IIa and BCL2, immunohistochemical staining was performed using anti-MyHC-I antibody (sc-66980: Santa Cruz Biotech, Santa Cruz, CA, USA), anti-MyHC-IIa antibody (sc-71632: Santa Cruz) and anti-BCL2 antibody (sc-492: Santa Cruz). Paraffin-embedded tissues from rat masseter muscles were prepared. For antigen retrieval, sections were incubated in trypsin for 7 min at 37 °C. The primary antibody dilutions were as follows: MyHC-I, MyHC-IIa and BCL2 for 1:50. The immunohistochemical procedures were performed as described in previous publication.[51] The negative controls were sections stained without primary antibodies. A total of 90 slides were fabricated and examined.

Stained sections were examined in an Olympus BX51 (Olympus, Tokyo, Japan) microscope. To quantify the immunohistochemical reaction intensity, the positive intensity immuno-staining in 10 random fields at ×100 magnification in the masseter muscle was evaluated by computer-assisted image analysis after image transformation to grayscale. The staining intensity was expressed as the mean intensity value (0: lowest intensity, 255: highest intensity). The samples were not counterstained so that the absorbance would be solely attributable to the product of the immunohistochemical reaction.

Transmission electron microscopic (TEM) analysis

The specimen preparation was referred to Cheongwon Center, KSBI. The detailed procedure was as follows. The masseter muscles were cut as 1 mm x 1 mm x 1 mm. These specimens were put into 2.5% glutaraldehyde in 1M PB buffer for overnight. The specimens were washed with 0.1M PB buffer for 10 min three times. After removing supernatant solution, they were put into 1% OsO_4 for 1h. The specimens were washed with 0.1M PB buffer for 10 min three times, again. The specimens were washed with distilled water for 5 min two times. Then, the specimens were underwent dehydration process with

a graded series of ethanol. The specimens substituted with 100% prophylene oxide for 30 min two times. Then, the specimens were embedded in prophylene oxide and Epon812 media. The embedded specimens were cut as ultra-thin section (70 nm thickness) using Ultra-Microtome (ULTRACUT UCT, LEICA, Installed at Korea Basic Science Institute). The prepared section was referred to Gangneung Center, KSBI for taking for TEM image. The cut section was placed on 150 mesh grid. The specimens were observed with JEM-2100F (JEOL, Japan, Installed at Korea Basic Science Institute) under 200keV.

Statistical analysis

SPSS for window ver. 19 (IBM Co., Armonk, NY, USA) were used for statistical analysis. The differences among groups were evaluated by ANOVA. For post-hoc tests, Bonferroni's method was used. The statistically significant level was set as $P<0.05$.

Ⅳ. RESULTS

1. Changes of food intake and body weight

There was no statistically significant difference among groups in the amount of food consumption from 2 weeks to 12 weeks after injection (Figure 1 and Table 1). The amount of food intake in rats treated with BTX (5 or 10 units) decreased significantly compared with control group only at 1 week after injection (P=0.032). There was no statistically significant difference among groups in the body weight during observation period (Figure 2 and Table 2). However, the variation in the group was high in BTX treated groups. The average value of body weight and the amount of food consumption were also generally lower in BTX treated groups compared with saline treated group (Figures 1 and 2). Particularly, the average value of body weight and the food consumption in the BTX treated groups were lower at 8 weeks after BTX injection. This was due to abnormal eruption of the incisors in some animals (2 animals for 5 U BTX treated group and 3 animals for 10 U BTX treated group). As the weight loss of the animal was so severe and life-threatening, the height of the incisors was reduced by grinding (Figure 2). After this procedure, the affected animals were recovered their body weight and food consumption rapidly. These findings were demonstrated in the observation at 10 weeks after injection (Figures 1 and 2).

2. Changes of muscle thickness

The muscle thickness and the relative muscle thickness at each observation point to the pre-injection level were shown in Table 3 and Figure 3. The BTX treated masseter muscles were significantly smaller than the saline injected masseter muscles from 2 weeks after injection. The average values of 5 U BTX group showed generally lower than those of 10 U BTX group. However, there was no statistically significant difference in muscle thickness between 5 U BTX treated group and 10 U BTX treated group during whole

observation periods (P>0.05). The thickness of the masseter muscle at 2 weeks after injection was 5.10 ± 0.22 mm, 3.43 ± 0.46 mm, and 3.76 ± 0.49 mm for the saline, 5 U, and 10 U BTX treatments, respectively (Table 3, P<0.001). The post-hoc test revealed differences between the groups treated with 5 U and 10 U BTX, resulting in significantly lower values compared with the saline treated control (P<0.001). The thickness of the masseter muscle at 12 weeks after injection was 5.77 ± 0.16 mm, 3.70 ± 0.36 mm, and 3.54 ± 0.60 mm for the saline, 5 U, and 10 U BTX treatments, respectively (P<0.001). The post-hoc test revealed differences between the groups treated with 5U and 10 U BTX, resulting in significantly lower values compared with the saline treated control.

3. Histomorphometric evaluation and immunohistochemical determination of MyHC-I, MyHC-IIa and BCL2

In the histological view, the muscle fibers in saline injected group had a normal polygonal shape, while the irregular and condensed degenerative changes of muscle fibers were evident in BTX treated groups at 2 weeks after injection (Figure 4). The space between myofibers was disappeared in BTX treated groups at 2 weeks after injection. It was partly recovered at 12 weeks after injection. The immunohistochemical findings demonstrated that the expression of MyHC-I was statistically significantly different among groups at 2 weeks after injection (Table 4, Figures 5 and 6, P=0.032). The relative expression of MyHC-I was increased in both BTX treated groups at 2 weeks after injection. The expression level of MyHC-I was 84.41 ± 9.60, 105.23 ± 12.22, and 92.88 ± 10.58 in saline, 5 U BTX treated, and 10 U BTX treated group, respectively at 2 weeks after injection. In the post-hoc test, 5 U BTX treated group resulted in significantly higher values compared with the saline group (P=0.031).

The elevated MyHC-I expression was decreased at 12 weeks after injection (Figure 6). The expression level of MyHC-I was 83.17 ± 9.60, 85.74 ± 16.19, and 72.98 ± 7.02 in saline, 5 U BTX treated, and 10 U BTX treated group, respectively at 12 weeks after injection. The

difference among groups was not statistically significantly different (P>0.05). Interestingly, 10 U BTX treated group at 12 weeks after injection had much lower expression compared to the same group at 2 weeks after injection. When compared MyHC-I expression in 10 U BTX treated group at 12 weeks after injection to that at 2 weeks after injection, the difference between groups was statistically significant (P=0.008).

There was statistically significant difference among groups in MyHC-IIa expression at 2 weeks after injection (Table 5). Both BTX treated groups displayed higher MyHC-IIa expression than the saline treated group (Figures 7 and 8). The expression level of MyHC-IIa was 83.61 ± 4.90, 115.07 ± 22.77, and 125.57 ± 18.39 in saline, 5 U BTX treated, and 10 U BTX treated group, respectively at 2 weeks after injection. The difference among groups was statistically significantly different (P=0.006). In the post-hoc test, 5 U BTX treated group and 10 U BTX treated group resulted in significantly higher values compared with the saline group (P=0.040 and 0.007, respectively). However, there was no statistically significant difference among groups in MyHC-IIa expression at 12 weeks after injection (P>0.05). The expression level of MyHC-IIa was 82.79 ± 5.73, 89.57 ± 9.14, and 98.25 ± 11.82 in saline, 5 U BTX treated, and 10 U BTX treated group, respectively at 12 weeks after injection. Both BTX treated groups displayed similar level of MyHC-IIa expression to the saline treated group.

The immunohistochemical findings demonstrated that the expression of BCL2 was statistically significantly different among groups at 2 weeks after injection (Table 6, Figures 9 and 10, P=0.001). The relative expression of BCL2 was increased in both BTX treated groups at 2 weeks after injection. The expression level of BCL2 was 80.65 ± 6.07, 113.45 ± 12.75, and 113.08 ± 15.03 in saline, 5 U BTX treated, and 10 U BTX treated group, respectively at 2 weeks after injection. In the post-hoc test, 5 U and 10 U BTX treated group resulted in significantly higher values compared with the saline group (P=0.003 for both groups).

The elevated BCL2 expression was maintained at 12 weeks after injection (Figure 10). The expression level of BCL2 was 78.20 ± 8.26, 93.92 ± 12.40, and 120.30 ± 23.56 in saline, 5

U BTX treated, and 10 U BTX treated group, respectively at 12 weeks after injection. The difference among groups was statistically significantly different (P=0.005). In the post-hoc test, 10 U BTX treated group resulted in significantly higher values compared with the saline group (P=0.004).

4. Ultrastructural changes after BTX injection

Considering the profound myofilaments abnormalities observed in the masseter muscle of BTX treated rats, the morphology of mitochondria was compared in TEM images. Destruction of myofibrils was observed within 2 week of 10 U BTX treatment (Figure 11). However, its destruction was not prominent in 5 U BTX treatment at 2 weeks after BTX injection. At 12 weeks after BTX injection, the number of enlarged mitochondria was increased in 5 U BTX treatment group. Regional destruction of myofilaments was also found. This type of myofilament destruction was more extensive and abruption of Z-line was observed in 10 U BTX treatment group at 12 weeks after injection. In addition, many mitochondria showed swelling of all mitochondrial spaces and cristae alterations. These results indicate that BTX treatment could be associated with the mitochondrial pathway of apoptosis in the muscle fiber of the masseter muscle.

Ⅴ. DISCUSSION

1. Changes of food intake and body weight

Even though the average value of the amount of food consumption and body weight were generally lower in the BTX treated groups compared with saline treated group, there was no statistically significant difference among groups from 2 weeks to 12 weeks. The amount of food intake in rats treated with BTX decreased significantly compared with control group only at 1 week after injection. In our previous study, the relative amount of food intake in rats treated with BTX (5-10 U) decreased significantly compared with control group (saline injection) from day 2 to day 7. The recovery in food intake after BTX injection was taken approximately 10 days after injection. In this study, although BTX treated group showed significant reduction in the food intake at 1 week, after that it was similar throughout the whole period. The result is consistent with other studies that BTX injection into masseter muscle induced only brief periods of problems with chewing and the return of normal function after a short time.[35, 44] This might have been due to compensatory hyperfunction of the other masticatory muscles such as temporalis muscle or medial pterygoid muscle to adjust to the masticatory fuction.[20, 45]

Interestingly, abnormal overeruption of the incisors was found in 2 rats for 5 U BTX treated group and 3 rats for 10 U BTX treated group at 8 weeks after BTX injection. Because this abnormal overeruption had interfered with normal mastication, the amount of food consumption in the BTX treated groups at 8 weeks was more reduced unlike other observation period. As the weight loss of these rats was so severe and life-threatening, the height of the overerupted incisors was reduced by grinding using dental high speed hand piece. After this procedure, body weight and food consumption of the affected animals were recovered rapidly. Abnormal overeruption of the incisors might result from deviation of incisors and lack of normal attrition of incisors following masseter muscle atrophy after BTX injection. Previous study reported that unilateral removal of masseter muscle of rats resulted in deviation of incisors like our study.[50] This would be probably the result of

functional imbalance in the masticatory muscle system. The abnormal overeruption of the BTX treated rats did not appear in the short term study.[27] This overeruption was not found in the other studies concerning BTX injection into the masster muscle of rats, that might be due to short observation period or unilateral BTX injection instead of bilateral injection. Moreover, as every rat treated with BTX did not show severe abnormal overeruption of the incisors, the effect on incisors eruption after BTX injection into masseter muscle in rats might be quite a variation.

2. Changes of muscle thickness

Ultrasound measurements of the thickness of the masseter muscle were performd to observe the muscle atrophy every week after BTX injection. Sonography is effective for imaging soft tissues of the body like muscles. Although muscle thickness measured by sonography does not directly mean the volume or mass of muscle, it can be useful to evaluate the extent of muscle atrophy after BTX injection clinically.[9] Furthermore, as serial measuring the muscle thickness by using sonography is possible without sacrificing or harming rats, continuous changes of muscle like atrophy and recovery can be identified.

The BTX-treated masseter muscles were significantly smaller than the saline solution–injected masseter muscles from 2 weeks after injection. There was no statistically significant difference in muscle thickness between 5 U BTX treated group and 10 U BTX treated group during whole observation periods. At 6 weeks, the atrophy of masseter muscle was maximal so that relative muslce thickness to the pre-injection level is 50.4% for 5U BTX group and 52.7% for 10U BTX group. This reduction was somewhat more than thickness/volume reductions of 6%-40% ranges from other studies.[20, 22, 52] This might be due to differences in the measurement method for thickness, mass or volume. Another reason was that previous other studies measured the muscle only at one or two paticular point, measured muscle atrophy may have not been the most severe period.

Muscle thickness in the BTX treated groups was gradually increasing after 6 weeks, but it was still significantly smaller than saline injected masseter muscle at 12 weeks. It was thought that a longer time than 12 weeks was required for BTX injected masseter muslce to recover completely. This result was similar to previous other studies. Rabbit masseters were still 8% lighter on average than the uninjected side at 12 weeks.[20] Other clinical human studies consistently show thickness/volume reductions of approximately 30% at 3 months after BTX treatment.[40, 44] Approximately half of the single injection patient population still shows masseter atrophy at 12 months.[9] It is not clear why volume recovery is delayed, nor whether it is ever fully normalized. One possibility is that some neurons or muscle fibers do not survive the procedure, resulting in a permanent loss of muscle mass. Another possibility is alteration of fiber type; because BTX tends to make muscle fibers slower, they may become smaller.[20] To confirm the hypotheses, the immuonohistochemical analysis to determine the alteration of muscle fiber type and ultrastructural study on muscle fibers using a transmission electron microscopy were required, and they will be discussed later.

3. Changes of muscle fibers after BTX injection

The force–velocity properties of muscle fibers, responsible for the differentiated muscle functions, are mainly dependent on their myosin heavy chain (MyHC) composition.[53] Myosin is a highly conserved, ubiquitous protein found in all eukaryotic cells, where it provides the motor function for diverse movements such cytokinesis, phagocytosis and muscle contraction.[54] There are several MyHC isoforms which can be classified according to their contraction speed in fast type (MyHC-IIb, MyHC-IIx and MyHC-IIa) and slow type (MyHC-I).[53-55] Many previous studies showed that BTX caused shifts in myosin heavy chain composition in muscle. Except for some research,[45] most studies of the influence of BTX found similar results for adult rat gastrocnemius,[56, 57] laryngeal,[58] and ocular medial rectus muscle.[59] No matter what kind of muscle was tested, the MyHC isoform tends to shift from IIb to IIa, IIx, and I. The adaptation to toxin paralysis (shift toward more slow

myosin isoforms) is the result of new neurons innervating the muscle.[57] MyHC type I and type IIa in the rat masseter muscle were also increased statistically significantly in both BTX treated groups at 2 weeks after injection. In this study, the change of MyHC type IIb was not tested regrettably but it coud be assumed that MyHC type IIb would decrease correspondingly. Additional experiments are needed to confirm this assumption. There was no significant difference in the changes of muscle fibers composition between 5 U BTX treated and 10 U BTX treated groups at 2 weeks.

At 12 weeks after BTX injection, there was no statistically significant difference among 3 groups in MyHC type I and type IIa. However, in the study of BTX injection into rat gastrocnemius muscles, the myosin heavy chain composition remained distinctly different from that of the control group after 12 weeks.[56] In another study, the MyHC expression in BTX-paralyzed ocular medial rectus muscle of rats shifted toward slower isoforms and did not normalize, even after 8 months.[59] It was supposed that it was due to the difference in the recovery reaction of different kind of muscle or different dosage of BTX. To explain why muscle volume recovery after BTX treatment is delayed, nor whether it is ever fully normalized, it was guessed that BTX tends to make muscle fibers slower, therefore, they may become smaller. However, this hypothesis could not be accepted at least in the masseter muscle of rats, because there was no significant difference in the MyHC composition between BTX treatment group and saline injection group at 12 weeks.

4. Ultrastructural changes after BTX injection

At 2 weeks after injection, destruction of myofilbrils could be found only in the 10 U BTX group. But this dissolution of myofibrils in the 10 U BTX group was not severe. In the prevous study, most fibres appeared normal in the masseter muscle of adult monkeys at 2 weeks after BTX injection.[24] However, muscle fibres in the masseter muscle of monkeys showed myofibrillar dissolution, aberrations in the Z-line, and enlarged mitochondria in the region of the I-band at 4 weeks. At 12 weeks after injection, both 5 U and 10 U BTX

group showed destruction of myofilaments. Especially, more extensive and severe destruction could be found in the 10 U BTX group. This result suggested that the extent of myofibrillar destruction might be associated with the injected BTX dose. Nonetheless, there was no statistically significant difference in muscle thickness between 5 U and 10 U BTX treated group during whole observation periods.

Interestingly, BTX treated groups showed mitochondrial swelling only at 12 weeks. Especially in 10 U BTX treated group, more severe alteration of cristae could be found. Although the major function of mitochondria is the aerobic production of ATP in cells, these organelles are also involved in many other important cellular functions. For example, in muscle fibers, mitochondria play an important role in the regulation of myonuclear apoptosis.[60] Many previous studies confirmed that prolonged skeletal muscle inactivity results in altered mitochontrial morphology, mitochondrial respiratoy dysfuction, and increased mitochondrial reactive oxygen species (ROS).[61-63] Moreover, denervation-induced muscle atrophy is also associated with deleterious changes in the mitochondrial protein import system along with increased mitochondrial susceptibility to apoptosis.[61, 63] BTX injection into masseter muscle of rats causes muscle paralysis with the inhibition of neurotransmitter release in the neuromuscular junctions, therefore similar ultrastructural changes like that could be found in the denervation-induced muscle atrophy was expected and confirmed in this study. In addition, this changes were associated with the dosage of BTX injected.

BCL2 (B-cell lymphoma 2), also known as Bcl-2, belongs to the Bcl-2 family. Bcl-2 family proteins regulate and contribute to programmed cell death or apoptosis. It is expressed in a variety of tissues. It regulates cell death by controlling the mitochondrial membrane permeability and inhibits caspase activity either by preventing the release of cytochrome c from the mitochondria and/or by binding to the apoptosis-activating factor.[64, 65] In the immunohistochemical study, the expression level of BCL2 in the both 5 U and 10 U BTX treated group was significantly higher than that in the saline group at 2 weeks after injection. At 12 weeks, the expression level of BCL2 in the 10 U BTX group was more increased,

significantly higher than that in the saline group. Changes of BCL2 expression level and altered mitochondrial morphology after BTX injection into masseter muscle suggest that the mitochondrial pathway of apoptosis in the muscle fiber might be associated with BTX treatment. In the TEM images, the mitochondrial swelling and alteration of cristae were prominent in the 10 U BTX group after 12 weeks. Therefore, BTX injection to the masseter muscle of rats with a high dosage might be associated with increased mitochondrial susceptibility to apoptosis as a delayed phenomenon. Howevere, it is considered that further studies are neeeded to investigate how the regulating proteins associated with apoptosis such as BCL2 acting in response to BTX treatment into masseter muscle.

VI. CONCLUSIONS

1. Bilateral BTX injection to the masseter muscle of rats had little effect on the body weight and the amount of food consumption in the long term. However, BTX treated group showed a great deal of variability between individual rats in the body weight changes and the amount of food consumption.

2. The maximal atrophy of masseter muscle after BTX injection was found at 6 weeks. A longer time than 12 weeks was required for BTX injected masseter muscle to recover completely. There was no significant difference in muscle thickness according to the injected dose of BTX.

3. The expression level of MyHC type I and type IIa in the BTX treated masseter muscles was significantly increased at 2 weeks. However, the expression level of MyHC type I and type IIa was not different from that of the control group after 12 weeks regardless of the injected dose of BTX.

4. The mitochondrial swelling and alteration of cristae were prominent in the 10 U BTX group after 12 weeks. Therefore, BTX injection to the masseter muscle of rats with a high dosage might be associated with increased mitochondrial susceptibility to apoptosis as a delayed phenomenon.

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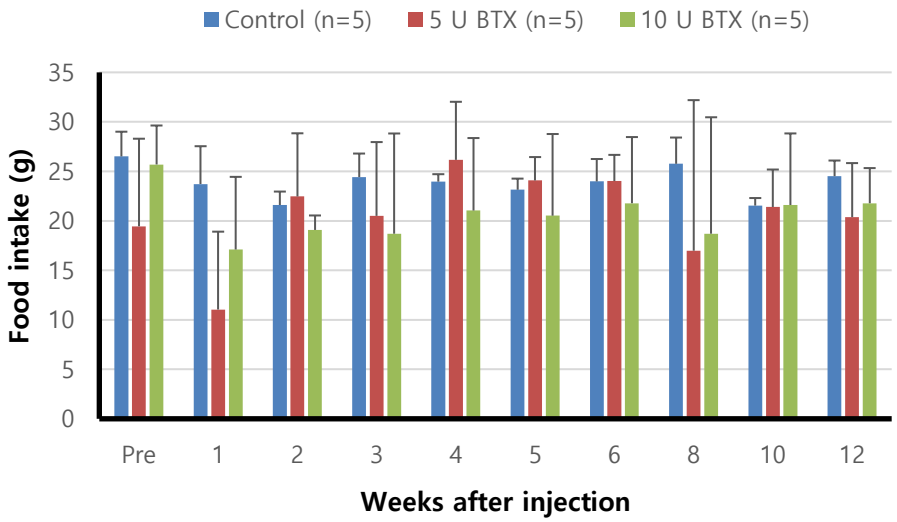

Figure 1. Food intake until 12 weeks after injection. There was no statistically significant difference among groups in the amount of food consumption during observation period except 1 week after injection.

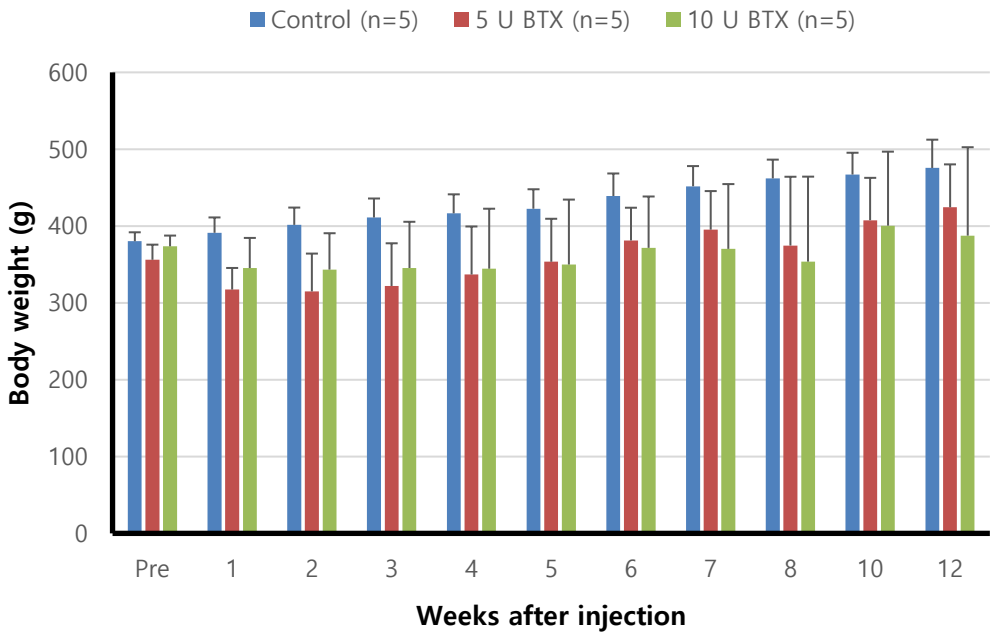

Figure 2. Body weight change after injection. Although the average value of body weight was generally lower in BTX (5 or 10 units) treated groups compared with control group, there was no statistically significant difference among groups in the body weight during observation period.

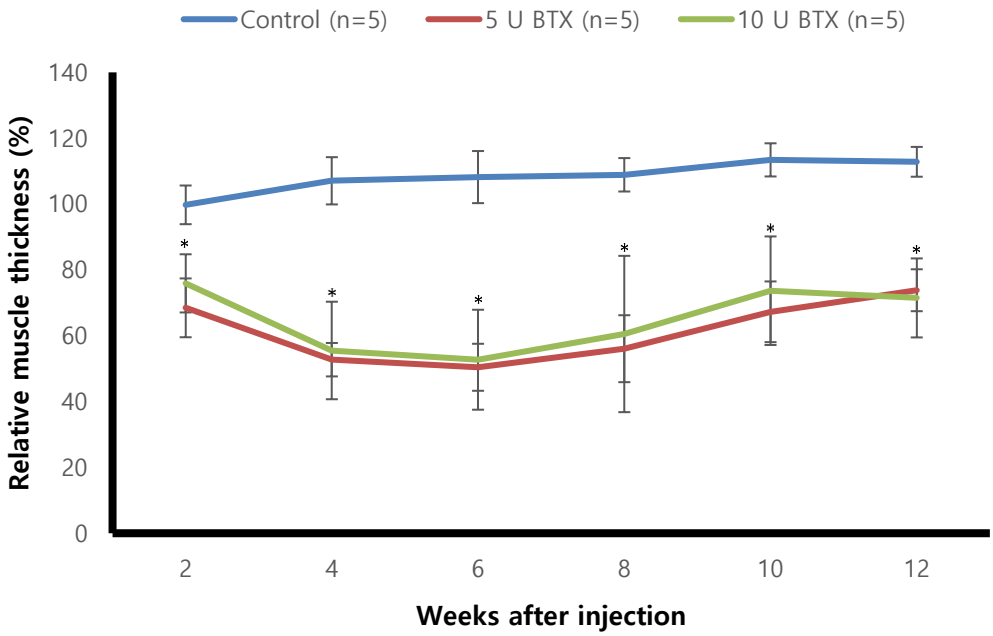

Figure 3. Muscle thickness measured by ultrasonography. The BTX (5 or 10 units) treated masseter muscles were significantly smaller than the saline injected masseter muscles from 2 weeks after injection (asterisk, $P<0.05$). However, there was no statistically significant difference in muscle thickness between 5 U BTX treated group and 10 U BTX treated group during whole observation periods ($P>0.05$).

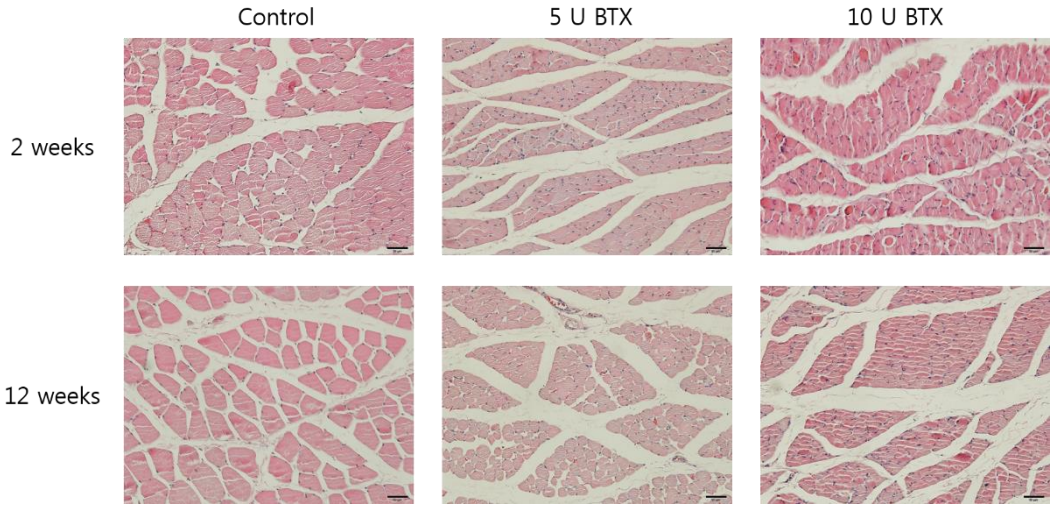

Figure 4. Histological views. The muscle fibers in saline injected group had a normal polygonal shape, while the irregular and condensed degenerative changes of muscle fibers were evident in BTX treated groups.

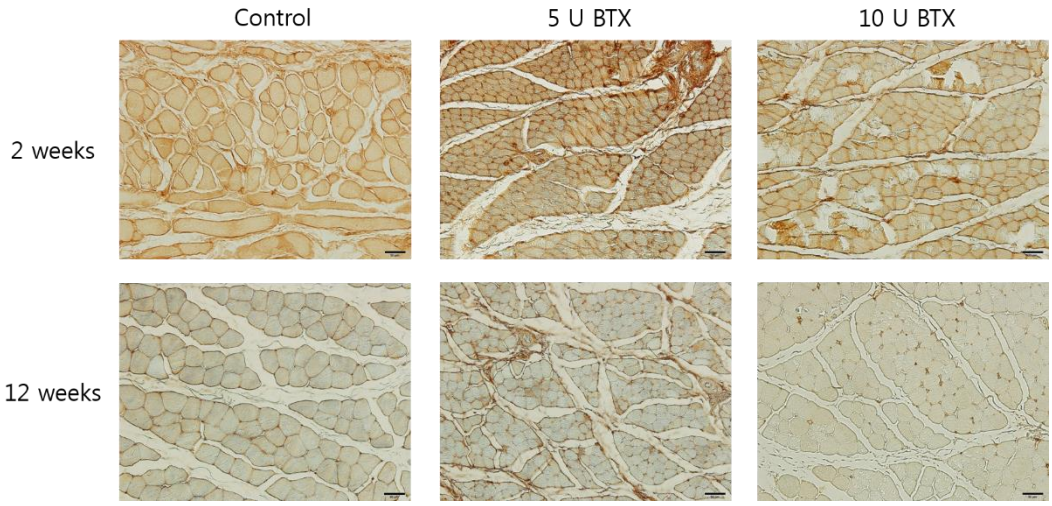

Figure 5. Immunohistochemical findings of myosin heavy chain type I. The expression of MyHC-I was increased in both BTX treated groups at 2 weeks after injection. The elevated MyHC-I expression was decreased at 12 weeks after injection.

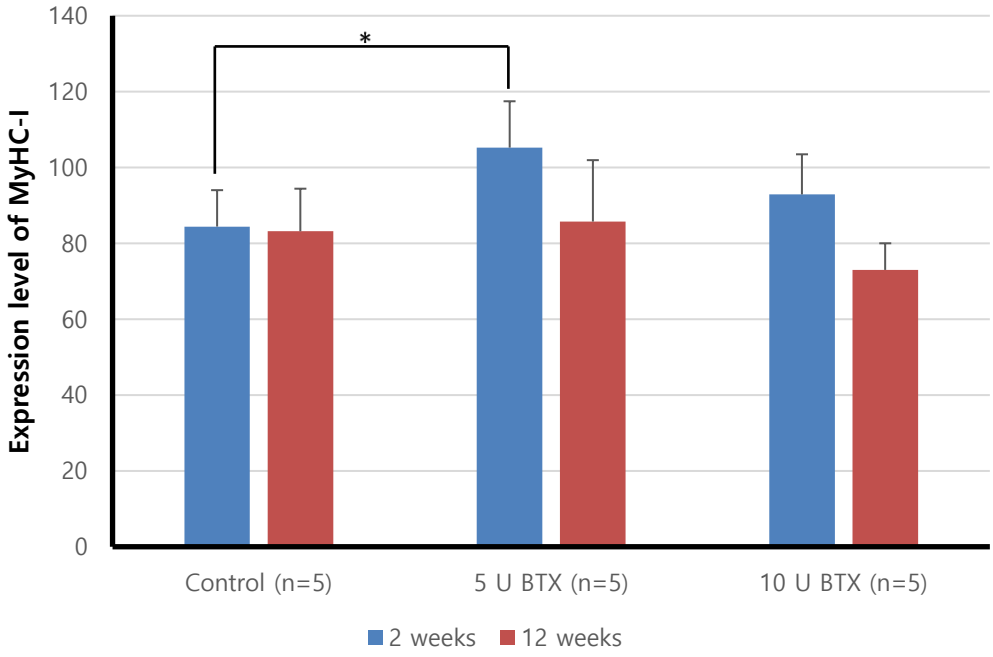

Figure 6. The expression level of myosin heavy chain type I (MyHC-I) in each group. The 5 U BTX treated group resulted in significantly higher values compared with the control group at 2 weeks after injection (asterisk, $P<0.05$, result of ANOVA).

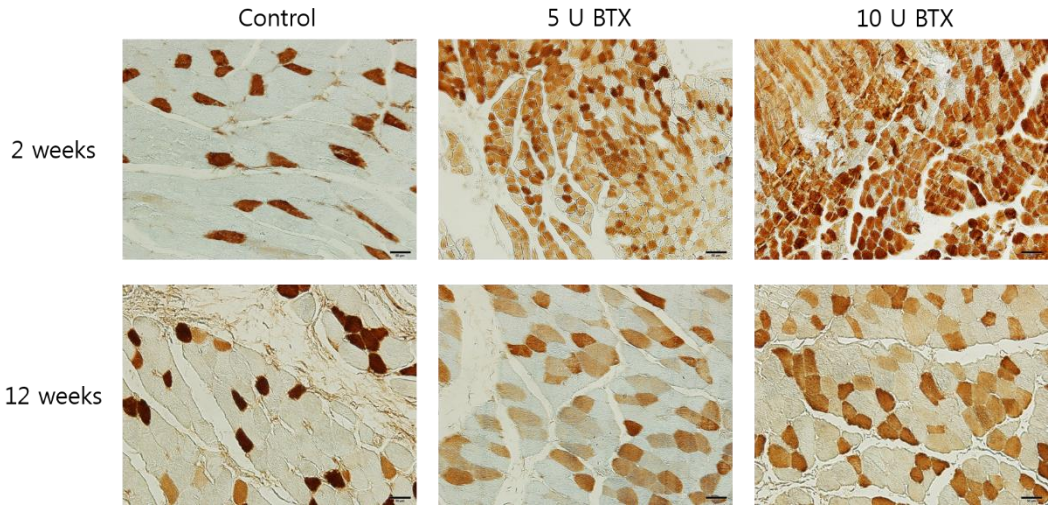

Figure 7. Immunohistochemical findings of myosin heavy chain type IIa. Both BTX treated groups displayed higher MyHC-IIa expression than the control group at 2 weeks after injection. However, both BTX treated groups displayed similar level of MyHC-IIa expression to the control group at 12 weeks after injection.

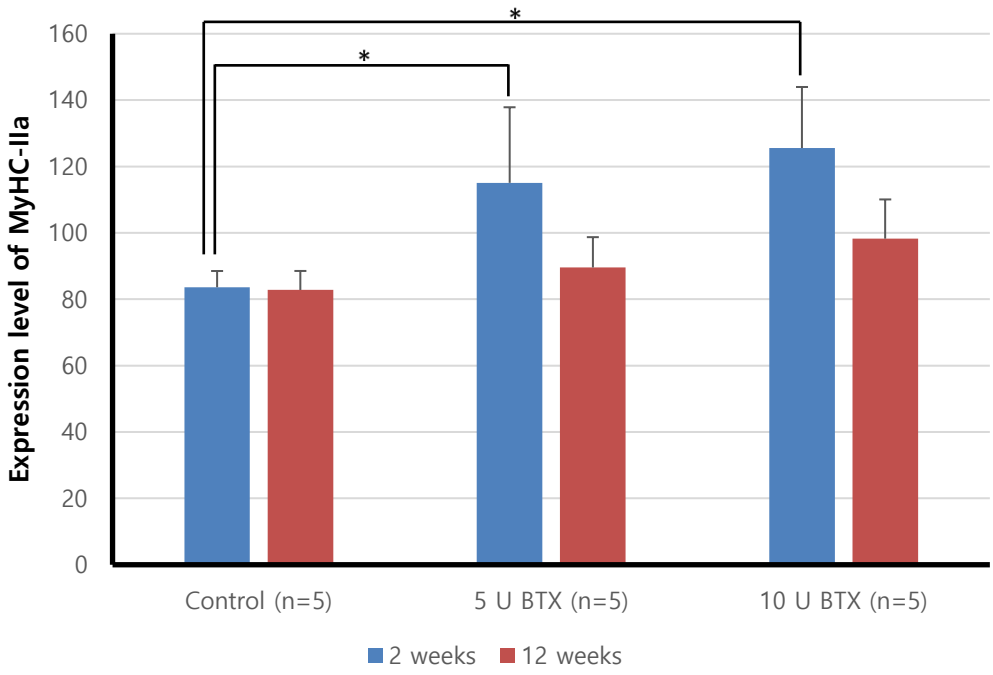

Figure 8. The expression level of myosin heavy chain type IIa (MyHC-IIa) in each group. The 5 U BTX treated group and 10 U BTX treated group resulted in significantly higher values compared with the control group at 2 weeks after injection (asterisk, $P<0.05$, result of ANOVA).

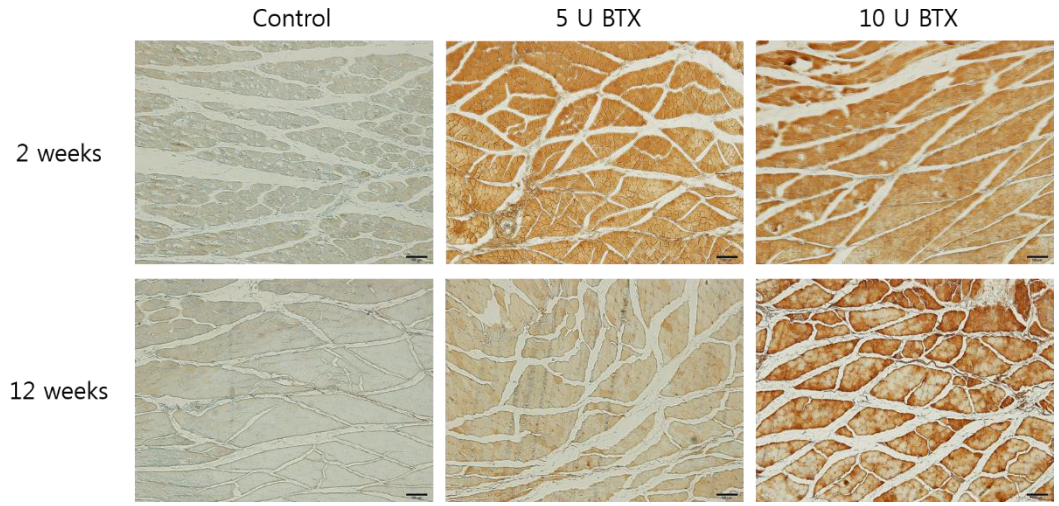

Figure 9. Immunohistochemical findings of BCL2. The expression of BCL2 was increased in both BTX treated groups at 2 weeks after injection. The elevated BCL2 expression in the 10 U BTX treated group was maintained at 12 weeks after injection.

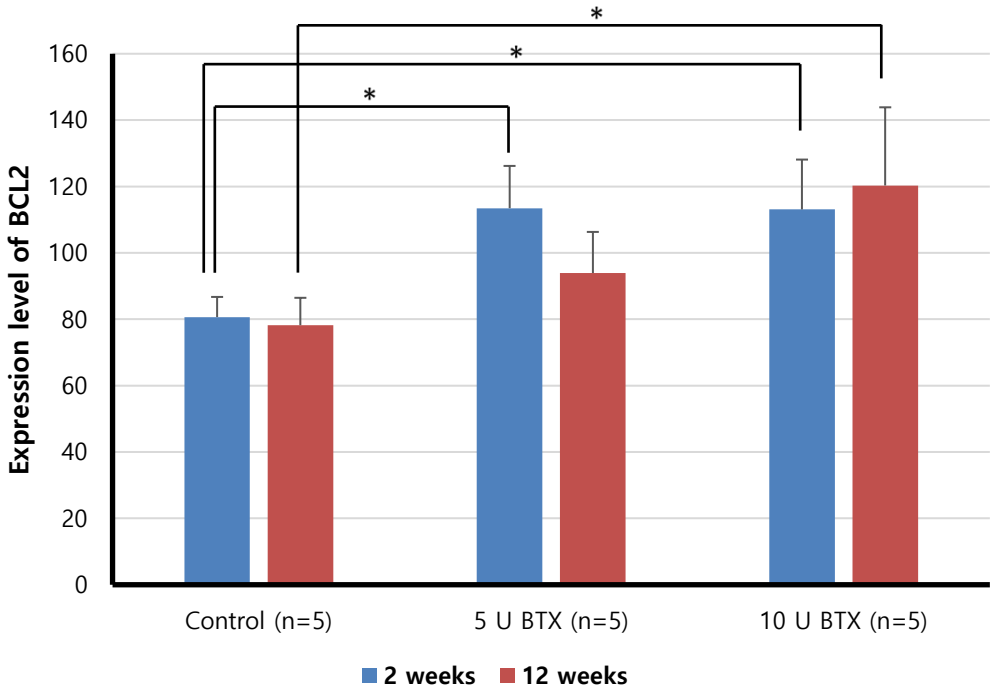

Figure 10. The expression level of BCL2 in each group. The 5 U BTX treated group and 10 U BTX treated group had significantly higher values compared with the control group at 2 weeks after injection (asterisk, $P<0.05$, result of ANOVA). The 10 U BTX treated group had significantly higher values compared with the control group at 12 weeks after injection (asterisk, $P<0.05$, result of ANOVA).

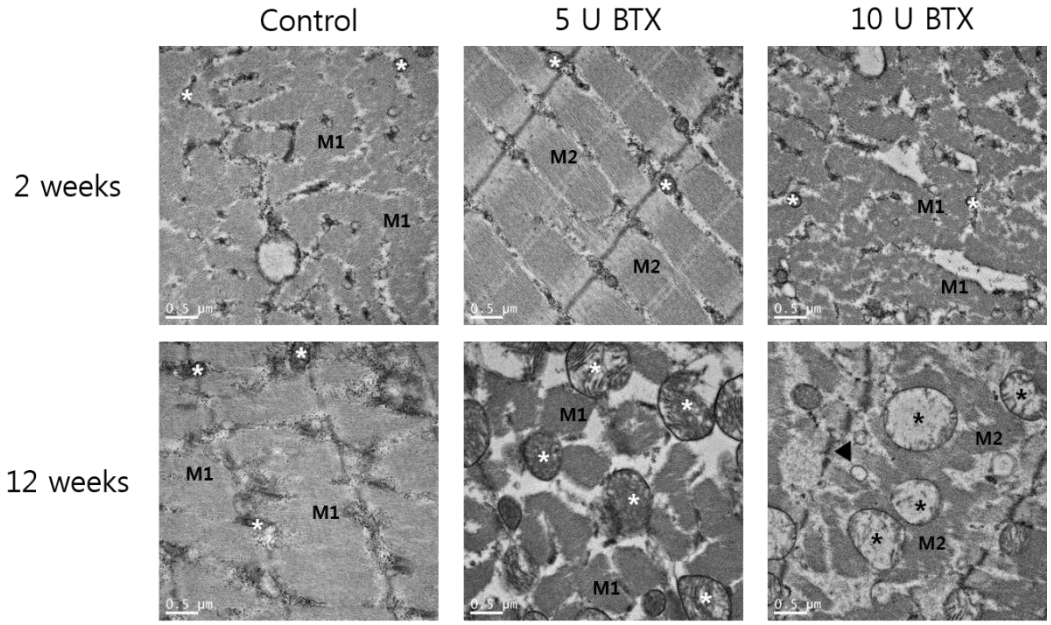

Figure 11. TEM images of each group. Myofilaments (M1, myofilaments in cross-section; M2, myofilaments in longitudinal section) were degenerated in BTX treated group. Mitochondria (*) were dramatically changed after BTX injection. Mitochondrial enlargement (*) was evident in the samples of 12 weeks after injection. Mitochondria having aberrant cristae were more common in the 10 U BTX treated group than in the other groups. In addition, abruption of Z-line (arrowhead) was observed in the 10 U BTX treated group.

Table 1. Food intake (g) until 12 weeks after injection

Weeks after injection	Control (n=5)		5 U BTX (n=5)		10 U BTX (n=5)		P value
	Mean	SD	Mean	SD	Mean	SD	
Pre	26.5	2.5	19.4	8.8	25.7	3.9	0.149
1	23.7	3.8	11.0	7.9	17.1	7.3	0.032*
2	21.6	1.4	22.5	6.4	19.1	1.5	0.385
3	24.4	2.4	20.5	7.5	18.7	10.1	0.479
4	24.0	0.8	26.2	5.9	21.0	7.3	0.359
5	23.1	1.1	24.1	2.4	20.5	8.3	0.525
6	24.0	2.3	24.0	2.6	21.8	6.7	0.658
8	25.8	2.7	17.0	15.2	18.7	11.8	0.447
10	21.5	0.8	21.4	3.8	21.6	7.2	0.998
12	24.5	1.6	20.4	5.5	21.8	3.6	0.267

*Significant at $P < 0.05$, result of ANOVA.

SD, standard deviation.

Table 2. Body weight (g) after injection

Weeks after injection	Control (n=5)		5 U BTX (n=5)		10 U BTX (n=5)		P value
	Mean	SD	Mean	SD	Mean	SD	
Pre	380.2	11.6	356.1	19.7	373.6	14.0	0.075^{NS}
1	391.2	20.0	317.3	28.1	345.3	39.2	0.053 ^{NS}
2	401.5	22.5	314.9	49.3	343.3	47.3	0.062 ^{NS}
3	411.1	24.8	322.0	55.6	345.4	60.1	0.069 ^{NS}
4	416.5	24.8	336.9	62.5	344.5	78.1	0.106 ^{NS}
5	422.5	25.4	353.5	56.0	349.9	84.5	0.143 ^{NS}
6	439.1	29.3	381.2	42.6	371.6	66.9	0.101 ^{NS}
7	451.7	26.3	395.3	50.2	370.3	84.3	0.121 ^{NS}
8	462.0	24.4	374.6	89.5	353.7	110.6	0.135 ^{NS}
10	467.0	28.4	407.5	55.2	400.5	96.3	0.257 ^{NS}
12	475.6	36.8	424.3	56.0	387.5	115.1	0.232 ^{NS}

NS, Not significant, result of ANOVA.

SD, standard deviation.

Table 3. Muscle thickness (mm) measured by ultrasonography

Weeks after injection	Control (n=5)		5 U BTX (n=5)		10 U BTX (n=5)		P value	Multiple comparisons[a]
	Mean	SD	Mean	SD	Mean	SD		
Pre	5.12	0.21	5.01	0.20	4.95	0.13	0.167	C = 5U = 10U
2	5.10	0.22	3.43	0.46	3.76	0.49	<0.001*	C > 5U = 10U
4	5.48	0.40	2.64	0.26	2.75	0.74	<0.001*	C > 5U = 10U
6	5.54	0.48	2.53	0.40	2.61	0.77	<0.001*	C > 5U = 10U
8	5.57	0.24	2.81	0.53	3.00	1.20	<0.001*	C > 5U = 10U
10	5.80	0.18	3.37	0.48	3.65	0.84	<0.001*	C > 5U = 10U
12	5.77	0.16	3.70	0.36	3.54	0.60	<0.001*	C > 5U = 10U

* Significant at $P < 0.05$, result of ANOVA.

[a] Bonferroni's multiple comparison test at the significance level of $P < 0.05$.

SD, standard deviation; C, Control; 5U, 5 U BTX; 10U, 10 U BTX.

Table 4. The expression level of myosin heavy chain type I (MyHC-I)

Weeks after injection	Control (n=5)		5 U BTX (n=5)		10 U BTX (n=5)		P value	Multiple comparisons[a]
	Mean	SD	Mean	SD	Mean	SD		
2	84.41	9.60	105.23	12.22	92.88	10.58	0.032[*]	C < 5U
12	83.17	11.23	85.74	16.19	72.98	7.02	0.250	C = 5U = 10U

[*] Significant at $P < 0.05$, result of ANOVA.

[a] Bonferroni's multiple comparison test at the significance level of $P < 0.05$.

SD, standard deviation; C, Control; 5U, 5 U BTX; 10U, 10 U BTX.

Table 5. The expression level of myosin heavy chain type IIa (MyHC-IIa)

Weeks after injection	Control (n=5)		5 U BTX (n=5)		10 U BTX (n=5)		P value	Multiple comparisons[a]
	Mean	SD	Mean	SD	Mean	SD		
2	83.61	4.90	115.07	22.77	125.57	18.39	0.006[*]	C < 5U = 10U
12	82.79	5.73	89.57	9.14	98.25	11.82	0.063	C = 5U = 10U

[*] Significant at $P < 0.05$, result of ANOVA.

[a] Bonferroni's multiple comparison test at the significance level of $P < 0.05$.

SD, standard deviation; C, Control; 5U, 5 U BTX; 10U, 10 U BTX.

Table 6. The expression level of BCL2

Weeks after injection	Control (n=5)		5 U BTX (n=5)		10 U BTX (n=5)		P value	Multiple comparisons[a]
	Mean	SD	Mean	SD	Mean	SD		
2	80.65	6.07	113.45	12.75	113.08	15.03	0.001*	C < 5U = 10U
12	78.20	8.26	93.92	12.40	120.30	23.56	0.005*	C < 10U

* Significant at $P < 0.05$, result of ANOVA.

[a] Bonferroni's multiple comparison test at the significance level of $P < 0.05$.

SD, standard deviation; C, Control; 5U, 5 U BTX; 10U, 10 U BTX.

국문초록

백서의 교근에 보툴리눔 독소 A 주사 시 단기 및 장기간의 영향: 면역조직학적 및 초미세구조 연구

문 영 민

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(지도교수 : 김 태 우)

연구 목적: 사람의 두경부 영역에서 저작근에 보툴리눔 독소 A (Botulinum toxin A, BTX) 주사는 개구장애, 이갈이, 이악물기, 편두통, 악관절 장애, 교근 비대 등에 사용되고 있다. 근거에 입각한 BTX의 활용을 위해서는 잘 설계된 randomized controlled trial (RCT)와 BTX 주사 후 장기간에 걸친 반응과 회복에 대한 기초적인 연구가 더욱 필요하다. 이번 동물 실험 연구는 교근에 양측성으로 BTX를 주사하여 단기 및 장기간에 걸쳐 BTX 용량에 따른 근육의 위축과 회복 양상을 비교 연구하고자 한다.

재료 및 방법: 총 30마리의 성숙한 수컷 백서를 준비하여 무작위로 3개의 군으로 나누었다. 각 군 백서의 교근에 양측성으로 생리식염수 (대조군), 5 유닛의 BTX, 10 유닛의 BTX를 각각 주사하였다. 주사 후 12주까지 하루 사료 섭취량과 체중을 측정하였고, 초음파를 이용하여 교근

의 두께를 매주 측정하였다. 실험에 사용된 백서의 절반은 2주 후, 나머지 절반은 12주 후에 희생시켜 교근에 대한 조직표본을 만들었다. 면역조직화학염색법을 통해 myosin heavy chain (MyHC) 구성의 변화를 분석하고, 투과 전자 현미경으로 초미세구조의 변화를 관찰하였다.

결과: 체중이나 사료섭취량은 2주부터 12주까지 관찰 기간 동안 세 군 사이에 통계적으로 유의할 만한 차이는 보이지 않았다. 교근에 BTX 주사 후 근육의 위축은 6주경에 가장 심하게 나타났으며, 완전히 회복하기 위해서는 12주 보다 긴 시간이 필요해 보였다. 주사한 BTX 용량에 따른 근육 두께의 유의한 차이는 보이지 않았다. BTX 주사 2주 후에 MyHC type I 과 type IIa 발현은 유의하게 증가하였으나, 12주 후에는 BTX 용량에 상관 없이 대조군과 유의한 차이를 보이지 않았다. 투과 전자 현미경을 통하여 BTX 주사 12주 후에 근원섬유의 이상을 발견할 수 있었다. 또한 10 유닛의 BTX를 주사한 군에서 12주 후에 미토콘드리아의 부종과 내막 주름(cristae)의 변화가 현저하게 나타났다.

결론: 백서의 교근에 BTX 주사 시 단기적으로 MyHC의 구성에 변화를 가져오지만, 12주 후에는 거의 원래 상태로 회복되었다. 백서의 교근에 고용량의 BTX 주사 시 미토콘드리아의 세포 자기 사멸(apoptosis) 감수성을 증가시키는 것과 장기적으로 관련이 있는 것으로 보인다.

주요어: 보툴리눔 독소 A, 교근, myosin heavy chain, 초미세구조 연구
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